In the Claims

Please amend the claims as shown below.

1. (Currently Amended) A method of ~~identifying~~ determining a nucleotide sequence of a nucleic acid comprising:

loading a first short sequencing reaction product at a first loading time into one or more lanes of ~~a~~ an electrophoresis sequencing ~~gel~~ device;

loading a second short sequencing reaction product onto the same one or more lanes of the sequencing ~~gel~~ device as the first short sequencing reaction product at a second loading time, wherein the first loading time and the second loading time are sufficiently temporally separated to separate the first short sequencing reaction product from the second short sequencing reaction product by electrophoresis; and

~~performing gel or capillary electrophoresis on~~ determining the sequence of the first short sequencing reaction product and ~~on~~ the sequence of the second short sequencing reaction product.

2. (Currently Amended) The method of claim 1, wherein the first short sequencing reaction product is produced from a region comprising a SNP (single nucleotide polymorphism).

3. (Currently Amended) The method of claim 1, wherein the first short sequencing reaction product is produced from an EST (expressed sequence tag).

4. (Previously Amended) The method of claim 1, wherein the first short sequencing reaction product and second short sequencing reaction product are each about 20 bases or shorter.

5. (Previously Amended) The method of claim 1, wherein the first short sequencing reaction product is a run off sequencing reaction product.

6. (Currently Amended) A method of determining the nucleotide sequence of a portion of a nucleic acid comprising:

a) isolating the nucleic acid from a nucleic acid library wherein the library comprises a recognition site of an enzyme that cuts at least 1 base downstream of the recognition site, wherein the recognition site is positioned within 1 base of an insert of the library;

b) amplifying the nucleic acid;

c) digesting the amplified nucleic acid with the enzyme;

d) performing a run-off sequencing reaction utilizing a primer that hybridizes to a region of an amplified fragment of said amplified and digested nucleic acid at or upstream of the recognition site to form a first sequencing reaction product;

e) loading a first sequencing reaction product at a first loading time into one or more lanes of an electrophoresis sequencing device; and

f) ~~performing electrophoresis analysis on~~ determining the sequence of the first sequencing reaction product.

7. (Currently Amended) The method of claim 6, further comprising the steps of:

g) loading a second sequencing reaction product onto the same one or more lanes of the ~~an~~ electrophoresis sequencing device as the first sequencing reaction product at a second loading time, wherein the first loading time and the second loading time are sufficiently temporally separated to separate the first sequencing reaction product from the second sequencing reaction product by electrophoresis; and

h) ~~and performing electrophoresis analysis on~~ determining the sequence of the second sequencing reaction product.

8. (Previously Amended) The method of claim 6, wherein the enzyme is a restriction enzyme.

9. (Currently Amended) The method of claim 8, wherein the restriction enzyme is ~~BpmI~~ BpmI

10. (Currently Amended)The method of claim 6, wherein the ~~electrophoresis performed is~~ sequence is determined using gel electrophoresis.

11. (Currently Amended) The method of claim 6, wherein the ~~electrophoresis is performed with~~ sequence is determined using a capillary apparatus.

12. (Canceled)

13. (Canceled)

14. (Currently Amended) A method of determining the nucleotide sequence of a portion of a nucleic acid comprising:

a) isolating the nucleic acid from a nucleic acid library wherein the library comprises a recognition site of an enzyme that cuts at least 1 base downstream of the recognition site, wherein the recognition site is positioned within 1 base of an insert of the library;

b) amplifying the nucleic acid;

c) digesting the amplified nucleic acid with the enzyme;

d) performing a run-off sequencing reaction utilizing a primer that hybridizes to a region of ~~the~~ an amplified fragment of said amplified and digested nucleic acid at or upstream of the recognition site to form a ~~first~~ sequencing reaction product;

e) ~~performing mass spectrophotometry on of the first~~ determining the sequence of the sequencing reaction product, wherein the sequence is determined using mass spectrophotometry.

Remarks

In the Office Action mailed 25 February 2003, the Examiner notes that the information disclosure statement filed 9 November 2001 does not include legible copies of documents 3-9. Legible copies of the documents are supplied herewith for the Examiner's consideration.

In the Office Action, the Examiner objects to claims 7 and 9 for informalities. The informalities are addressed in the amended claims.

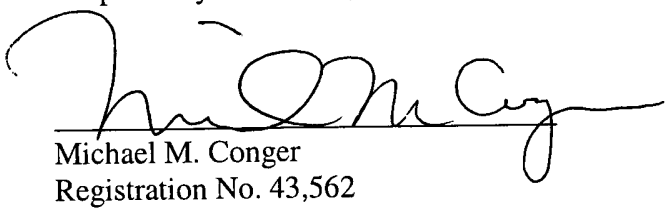
In the Office Action, claims 1-11 and 14 are rejected under 35 USC 112 second paragraph as being indefinite. The Examiner states that claims 1-5 and claims 6-11 are indefinite because the claims do not make clear whether performance of the final step in the claimed method, performing electrophoresis, is sufficient to meet the requirements of the claims. The Examiner also rejects claim 14 because the claim does not make clear whether performance of the final step in the claimed method is sufficient to meet the requirements of the claim. The Examiner requests clarification.

Claims 1, 6 and 14 have been clarified by amendment to indicate the final step of the claimed method is determining the sequence. Use of the term "amplified fragment" in claims 6 and 14, referring to a fragment of the amplified and digested nucleic acid, has been amended to clarify the meaning of the claim language.

Claims 1-11 and 14 are also rejected as being indefinite for use of unclear antecedents and the use of limitations for which there is insufficient antecedent basis. The Applicants have amended the claims to overcome these rejections.

Applicants respectfully submit that the claims as amended are in condition for allowance and request favorable reconsideration.

Respectfully Submitted,



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Non-radioactive automated sequencing of oligonucleotides by chemical degradation

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ABSTRACT

A non-radioactive sequencing of fluorescently labelled oligonucleotides by solid-phase chemical degradation is described. Although non-radioactive methods have been reported for the dideoxy chain termination technique, such a method has not yet been developed for the chemical degradation sequencing of DNA fragments. A 21-mer fluorescein labelled M13 sequencing primer was sequenced in an on-line automated system in about 30 minutes. The fluorescent dye and its bond to the oligonucleotide were stable during the chemical reactions used for the base specific degradations. As the sequence is determined on-line during electrophoresis, reloading and running 10 fragments simultaneously allows us to use one gel for sequencing of about 50 different oligonucleotides.

INTRODUCTION

During the last two years fully automated non-radioactive methods for DNA sequencing have been developed for the dideoxy chain termination procedures. The systems differ in their optical design and use either one dye (1, 2) or four different fluorescent dyes as labels for the four bases, which are attached via spacer groups either to the 5'-end of the primer (3, 4) or to the dideoxynucleoside triphosphates (5). After enzymatic chain elongation of the primer the products are separated by gel electrophoresis, the sequence is determined by automated systems during electrophoresis and stored directly in the computer. These methods have three principle advantages. First, gel handling, film exposure and reading of x-ray films are eliminated. These usually tend to be tedious and occupy a substantial part of the whole sequencing process. Second, the automated systems eliminate human errors in the reading. Third, work with hazardous and costly radiochemicals is avoided.

Non-radioactive methods have not yet been developed for the chemical degradation sequencing of DNA fragments, although several major improvements have been made recently to accelerate the chemical degradation. First, the introduction and use of solid phase carriers for the degradation procedures allows one to process many DNA fragments simultaneously. Consequently, the degradation process can be easily automated (6, 7). Second, new chemical sequencing vectors allow single-end labelling of DNA fragments (8).

Further increase in the speed of chemical degradation sequencing could be achieved by finding non-radioactive methods using a fluorescent dye as the label. The major problem is

expected to be the instability of the fluorescent dye during the chemical reactions used for the base-specific modification and degradation of the labelled DNA fragment. In this paper we report a technique for sequencing of fluorescein labelled oligonucleotides by solid-phase chemical degradation.

MATERIALS AND METHODS

Synthesis of oligomer and fluorescent labelling for sequence determination

Fully protected oligodeoxyribonucleotides were prepared on an Applied Biosystems DNA synthesiser using standard β-cyanoethyl phosphoramidite chemistry (9). A portion of the material was retained for a further synthetic cycle employing (S-trityl-3-mercapto-propyloxy), 2-cyanoethoxy N, N-diisopropylaminophosphine in the condensation step. This phosphoramidite was synthesised from S-trityl-3-mercaptopropanol in analogous fashion to the described methoxy compound (10).

After removal of blocking groups and cleavage from the support with ammonia, the S-trityl oligonucleotide was purified by reserved phase h.p.l.c. Detritylation with silver nitrate and subsequent reaction of the liberated thiol with 5-iodoacetamidofluorescein was carried out as described previously (2). The excess dye was removed by two ethanol precipitations of the labelled oligonucleotide. The fluorescein labelled oligodeoxy-ribonucleotide was then purified by reversed phase h.p.l.c. prior to sequencing by chemical degradation. For short oligonucleotides (<20 bases) the final h.p.l.c. purification can be omitted.

In the case described here, the oligonucleotide was a 21-mer, fluorescein labelled M13 sequencing primer of base sequence 5'-d[CGTTGTAAAACGACGGCCAGT].

Solid phase chemical degration

Chemical degration of oligonucleotides has been performed essentially as described in (7) using Hybond M & G paper (Amersham). We applied 5pmol of fluorescein labelled oligomer to the carrier in 1μl aliquots. For degradation, the following rections were used:

G:	with 1% DMS in 50mM ammonium formate buffer pH3.5 for 10 min.;
A+G:	with 80% formic acid for 20 min.;
T over purines: (T>Pu)	With 0.1mM $KMnO_4$ for 20 min.;
C:	with 4M hydroxylamine pH 6.

After piperidine reaction and lyophilization the samples were dissolved in 30% aqueous formamide (100μl).

Sequence determination

The base sequence in the DNA fragment was determined on-line in the automated system described previously (1, 2). After piperidine reaction and lyophilization the samples were dissolved in 30% aqueous formamide (100μl), and 1μl (corresponding to 0.05pmol of

the chemical reactions used for the
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h.p.l.c. Detritylation with silver
n 5-iodoacetamidofluorescein was
/e was removed by two ethanol
luorescein labelled oligodeoxy-
. prior to sequencing by chemical
final h.p.l.c. purification can be

21-mer, fluorescein labelled M13
GCCAGT].

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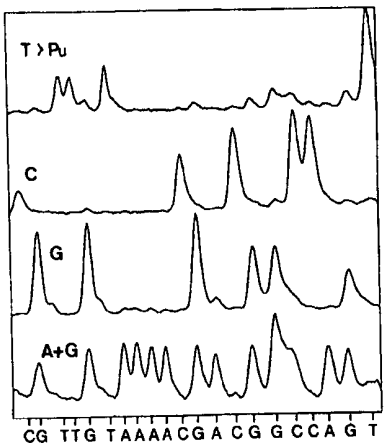

FIG. 1
Raw data output from the automated DNA sequencing system showing the sequence of the fluorescein labelled 21-mer d[CGTTGTAAAACGACGGCCAGT] obtained by the solid phase chemical degradation procedure. The peaks are normalised, i.e. the heights of the maxima in each track were set to be equal in the display. Chemical reactions were as described in materials and methods.

starting material) of each solution was loaded onto a 10% polyacrylamide gel containing 8M urea and run for about 30 minutes. A new set of degraded oligomers can be re-loaded on the sequencing gel after a short time (about 30 minutes) and sequenced. The same gel can be used for the sequence determination of up to 50 different fragments.

RESULTS AND DISCUSSION

The raw data from the sequencing run with chemical degradation of the fluorescently labelled primer (described above) determined in the automated DNA sequencing system (1, 2) is shown in Fig. 1. Determination of the sequence from the position of the peaks is straightforward as displayed in the figure. Although there are many secondary peaks in one track, they are much smaller than the significant ones. Observed heights of the significant peaks in one track are uniform, when compared with those obtained by the dideoxy method (1, 2) with Klenow enzyme, although other enzymes, like T7 DNA polymerase improve the uniformity (11). The peak uniformity, particularly beyond 250 bases is important for accuracy of the automated methods. In the Maxam-Gilbert procedure (Fig. 1) this observation allows easy discrimination of the weaker secondary peaks. Reading of the sequence starts from base number one, in contrast to the standard Maxam-Gilbert procedure using radioactive labels and degradation reactions in solution. The same gel can be used to sequence about 50 different DNA fragments, which represents a significant time saving compared to the standard methods

[redacted]

using radioactive labels, requiring new gel and film exposure for each electrophoresis run.

In conclusion, the fluorescent dye and its linkage to the oligonucleotide were sufficiently stable during the chemical reactions used for the base specific degradation to allow a non-radioactive sequence determination. The presence of weak secondary peaks, which is a normal accompanying feature of the Maxam-Gilbert method, and observed also by autoradiography, does not interfere with a reliable sequence determination.

Application of this method to much longer chemically synthesised oligodeoxyribonucleotides and an extension of the technique to encompass non-radioactive sequencing of longer DNA fragments will be reported in a subsequent publication.

The stability of the dye and linker to other commonly used degradative procedures that are compatible with solid phase sequencing, with an evaluation of the yield of the reactions and a study of the uniformity in peak heights will also be investigated and reported.

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